

# Evidence That a Hemoglobin Adduct Used for Dosimetry of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone Is a Carboxylic Ester

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Hemoglobin adducts of the carcinogenic tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) release 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) upon mild base or acid hydrolysis. HPB has been detected in hydrolysates of hemoglobin from smokers and snuff dippers and has been proposed as a dosimeter of exposure to and metabolic activation of NNK in people exposed to tobacco products. In this study, labeling experiments were carried out with [<sup>18</sup>O]NaOH that provide strong evidence that the globin adduct that releases HPB upon hydrolysis is a carboxylic ester. Globin was isolated from rats treated with [5-<sup>3</sup>H]NNK. This globin was reacted with NaCNBH<sub>3</sub>, followed by hydrolysis at room temperature with 0.2 N NaOH. Analysis of the products demonstrated the presence of 4-hydroxy-1-(3-pyridyl)-1-butanol, but not HPB. These results demonstrate that the adduct in globin has a free carbonyl group and is not a Schiff base. This sequence of reactions was then carried out with [<sup>18</sup>O]NaOH under conditions that were shown to result in incorporation of <sup>18</sup>O if nucleophilic displacement at C-4 had occurred. Analysis by GC-MS of the 4-hydroxy-1-(3-pyridyl)-1-butanol formed in this experiment demonstrated that there was no incorporation of <sup>18</sup>O. These results are consistent only with the hydrolysis of an ester by a B<sub>AC</sub>2 mechanism. Therefore, the adduct releasing HPB upon mild base hydrolysis must be a 4-(3-pyridyl)-4-oxobutyl ester of aspartate, glutamate, or a terminal carboxylate. Further support for this conclusion was obtained by investigating the chemistry of a model ester,  $\alpha$ -methyl- $\beta$ -4-(3-pyridyl)-4-oxobutyl-*N*-carbobenzyloxy-L-aspartate. The hydrolysis properties of this compound were similar to those of the HPB-releasing hemoglobin adduct.

## Introduction

When rats or mice are treated with the carcinogenic tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), hemoglobin adducts are formed (1–3). Mild base hydrolysis of this hemoglobin releases 20–40% of the bound material as 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB). Release of HPB from human hemoglobin has been suggested as a dosimeter of human exposure to, and metabolic activation of, NNK as well as a related nitrosamine, *N*'-nitrosoornicotine. Levels of HPB released from hemoglobin have been quantified in humans by GC-MS (4).

Figure 1 illustrates the postulated mechanism of NNK-hemoglobin adduct formation. Enzymatic hydroxylation of the methyl carbon yields  $\alpha$ -hydroxymethyl-NNK (compound 3 in Fig. 1), which is unstable and spontaneously decomposes to formaldehyde and 4-(3-pyridyl)-4-oxobutyl diazohydroxide (com-

pound 4 in Fig. 1). This intermediate reacts with a nucleophilic site in hemoglobin to produce an adduct that releases HPB upon hydrolysis with NaOH or HCl under mild conditions. Previous studies have provided support for this mechanism. These studies have shown that the adduct is covalently bound to globin (3).  $\alpha$ -Acetoxy-NNK (NNKOAc; compound 2 in Fig. 1), a stable precursor to  $\alpha$ -hydroxymethyl-NNK, formed the same adduct upon injection into mice (3). In contrast, HPB did not react with hemoglobin *in vitro* and did not produce HPB-releasing hemoglobin adducts when injected into mice (3).

An earlier study showed that cysteine is not a site of adduct formation in the hemoglobin of rats treated with NNK (5). In this paper, we describe experiments with [<sup>18</sup>O]NaOH that strongly support the hypothesis that the HPB-releasing hemoglobin adduct is a carboxylic ester.

## Results and Discussion

Our initial goal was to determine whether or not the adduct had a free carbonyl group. The cyclic intermediate (compound 6 in Fig. 2) is formed during the solvolysis of NNKOAc and could be involved in adduct formation, producing an adduct without a

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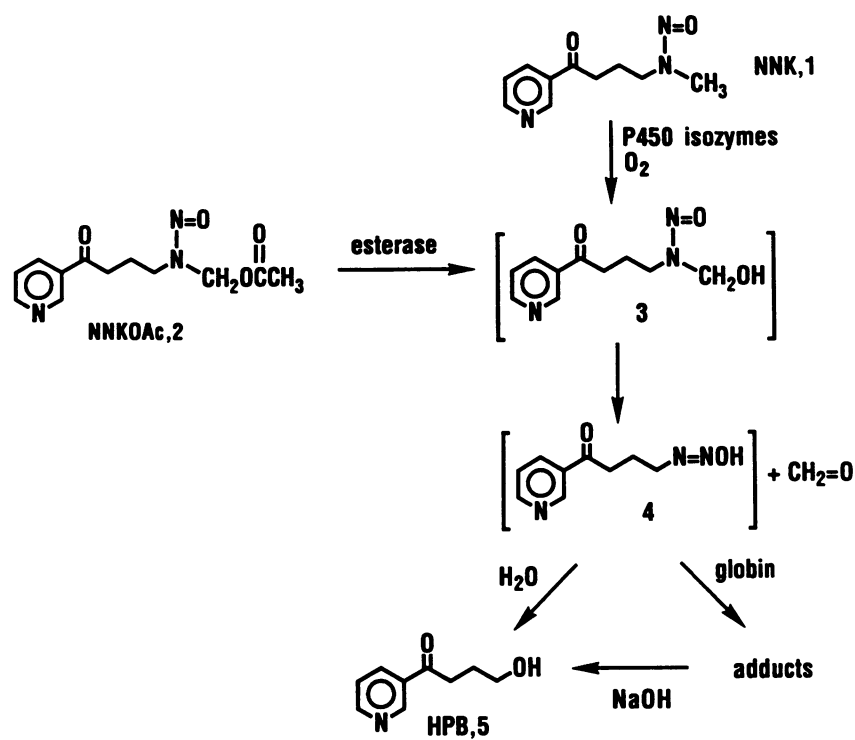


FIGURE 1. Metabolic activation of NNK to globin adducts that release HPB upon hydrolysis.

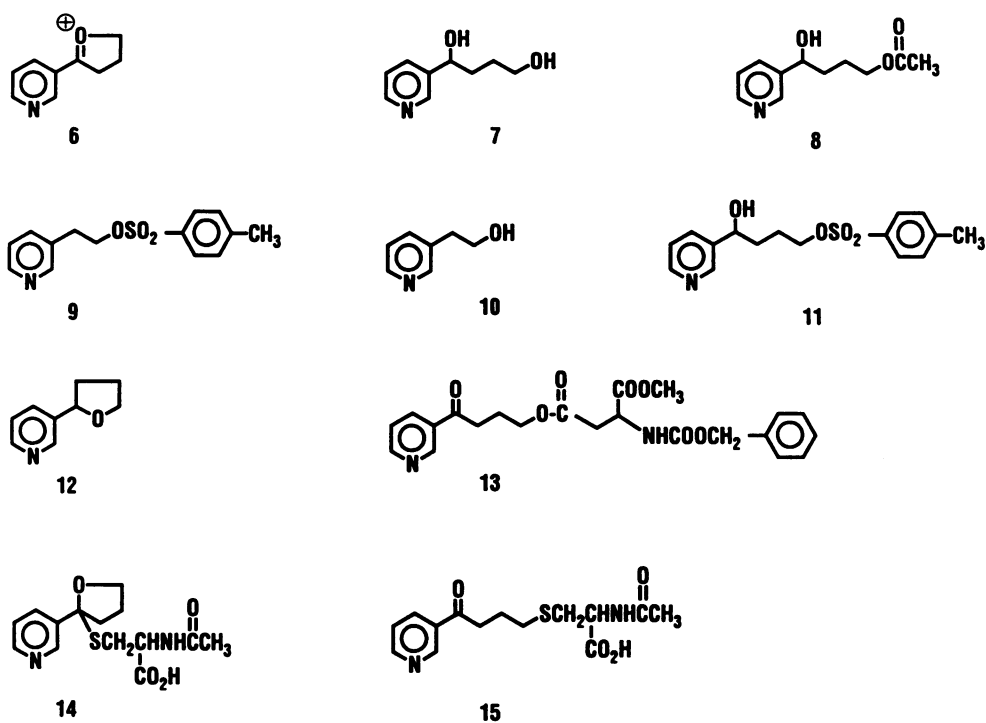


FIGURE 2. Intermediates, products, and model compounds related to NNK-globin adducts.

carbonyl group (6). This information was necessary before  $^{18}\text{O}$ -labeling experiments could be carried out, since they would be confounded by exchange if a free carbonyl group were present. Globin was isolated from rats treated with  $[5\text{-}^3\text{H}]\text{NNK}$ . The globin was allowed to react with  $\text{NaCNBH}_3$  at pH 6–7, conditions that do not release HPB. The  $\text{NaCNBH}_3$ -treated globin was dialyzed, then hydrolyzed with mild base. The hydrolysate was analyzed by HPLC. About 20% of the radioactivity co-eluted with 4-hydroxy-1-(3-pyridyl)-1-butanol (compound 7 in Fig. 2). HPB was not detected in this sample. When this sample was base treated without prior  $\text{NaCNBH}_3$  treatment, 25% of the radioactivity was released as HPB. These results demonstrate that a free carbonyl group is present in the HPB-releasing adduct, which therefore must be a 4-(3-pyridyl)-4-oxobutyl adduct rather than a cyclic one. The results also showed that the HPB-releasing adduct is not a Schiff base.

The next series of experiments involved hydrolysis with  $[^{18}\text{O}]\text{H}_2\text{O}$ . These experiments were carried out on globin that had been treated with  $\text{NaCNBH}_3$ , in order to avoid exchange of the carbonyl oxygen. If the adduct were a carboxylic ester, we would expect hydrolysis by the  $\text{B}_{\text{AC}}2$  mechanism and no incorporation of  $^{18}\text{O}$  into the diol (compound 7 in Fig. 2). Hydrolysis of an ether would not occur under the mild conditions that we are using. Any other adduct, between C-4 of the oxobutyl group and, for example, histidine or lysine of globin, would hydrolyze with incorporation of  $^{18}\text{O}$  into the diol.

Conditions for this experiment were developed using two model compounds, the hydroxy ester (compound 8 in Fig. 2) and the tosylate (compound 9 in Fig. 2). Compound 8 (Fig. 2) was a model for a carboxylic ester adduct. As expected, hydrolysis of this compound with  $[^{18}\text{O}]\text{NaOH}$  yielded compound 7 (Fig. 2), with an M+1 peak of 168 (relative intensity, 100) in its chemical ionization-MS; no peak at m/e 170 was detected. Thus, there was no incorporation of label into the diol (compound 7 in Fig. 2). Compound 9 (Fig. 2) was a model for adducts that would have undergone a nucleophilic substitution reaction resulting in incorporation of  $^{18}\text{O}$ . Hydrolysis with  $[^{18}\text{O}]\text{NaOH}$  produced mainly 3-vinylpyridine (95%). The minor product (compound 10 in Fig. 2) contained  $> 85\%$   $^{18}\text{O}$ . We also attempted to prepare hydroxy tosylate (compound 11 in Fig. 2) as a model, but it was unstable and its solvolysis produced exclusively the furan (compound 12 in Fig. 2). These control experiments established that our conditions were adequate to detect incorporation of  $^{18}\text{O}$  upon hydrolysis of the globin adduct.

Globin from rats treated with  $[5\text{-}^3\text{H}]\text{NNK}$  was reacted with  $\text{NaCNBH}_3$ , dialyzed, and hydrolyzed with  $[^{18}\text{O}]\text{NaOH}$  in  $[^{18}\text{O}]\text{H}_2\text{O}$ . Diol (compound 7 in Fig. 2) was isolated by HPLC, silylated, and analyzed by GC-MS. The molecular ion of the bis trimethylsilyl ether of the diol is m/e 311. The ratio of m/e 311 to m/e 313 was the same in the material isolated from globin as in the standard. Thus, there was no detectable incorporation of

$^{18}\text{O}$ , and the adduct must be a carboxylic ester.

Further evidence was obtained by synthesis of the model aspartate ester (compound 13 in Fig. 2). Hydrolysis was carried out under mild basic or acidic conditions. Release of HPB was complete in base, whereas only 6% was released in acid. The release of HPB from globin of NNK-treated rats under the same hydrolysis conditions was complete in base, accounting for 19.6% of the radioactivity bound to globin. In acid, only 2.3% of the bound radioactivity was released as HPB (5); thus, the extent of hydrolysis was 12% as great as that observed in base.

The carboxylic ester adduct of NNK in globin could be formed by reaction of diazohydroxide (compound 4 in Fig. 1) with aspartate, glutamate, or the terminal carboxyl groups of globin. A recent study has used 1-amino-2,3-propanediol to locate one of the benzo[a]pyrene diol epoxide carboxylic ester adducts at aspartate 47 of the  $\alpha$  chain of human hemoglobin (7). We are currently exploring this approach for identifying the acids involved in formation of the HPB-releasing adduct.

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